

TREATMENT OF METABOLIC DISORDERS
USING TNF α INHIBITORS

5 **Related Applications**

This application claims priority to prior filed U.S. Provisional Application Serial No. 60/397,275, filed July 19, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/411,081, filed September 16, 2002, and prior-filed U.S. Provisional Application Serial No. 60/417490, filed October 10, 2002.

- 10 This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/455777, filed March 18, 2003. In addition, this application is related to U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015. This application is also related to U.S. Patent Application Serial No. 09/801,185, filed March 7, 2001; U.S. Patent Application Serial No. 10/302,356, filed November 22, 2002; U.S. Patent Application Serial No. 10/163657, filed June 2, 2002; and U.S. Patent Application Serial No. 10/133715, filed April 26, 2002.

- This application is related to U.S. utility applications (Attorney Docket No. BPI-187) entitled "Treatment of TNF α -Related Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-188) entitled "Treatment of Spondyloarthropathies Using TNF α Inhibitors," (Attorney Docket No. BPI-189) entitled "Treatment of Pulmonary Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-190) entitled "Treatment of Coronary Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-191) entitled "Treatment of Metabolic Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-192) entitled "Treatment of Anemia Using TNF α Inhibitors," (Attorney Docket No. BPI-193) entitled "Treatment of Pain Using TNF α Inhibitors," (Attorney Docket No. BPI-194) entitled "Treatment of Hepatic Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-195) entitled "Treatment of Skin and Nail Disorders Using TNF α Inhibitors," (Attorney Docket No. BPI-196) entitled "Treatment of Vasculitides Using TNF α Inhibitors," (Attorney Docket No. BPI-197) entitled "Treatment of TNF α -Related Disorders Using TNF α Inhibitors," and PCT application (Attorney Docket No. BPI-187PC) entitled "Treatment of TNF α -Related Disorders," all of which are filed on

even date herewith. The entire contents of each of these patents and patent applications are hereby incorporated herein by reference.

Background of the Invention

5 Cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) are molecules produced by a variety of cells, such as monocytes or macrophages, which have been identified as mediators of metabolic processes. TNF α (also referred to as TNF) was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see *e.g.*, Old, L. (1985) *Science* 230:630-632). TNF α has been linked to
10 a number of metabolic disorders, including obesity and diabetes (Hotamisligil *et al.* (1993) *Science* 259:87). For example, TNF α has been linked to obesity in mice who show an elevated level of TNF expression in fat tissue (Hotamisligil *et al.* (1995) *J Clinl Invest.* 95:2409).

 There are several types of metabolic disorders and diseases associated with
15 human and animal metabolism, *e.g.*, type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic retinopathy, diabetic ulcerations, retinopathy ulcerations, neuropathy, peripheral neuropathy, diabetic macrovasculopathy and obesity. Diabetes mellitus is among the most common of all metabolic disorders, affecting up to 11% of the population by age 70. Type I (insulin dependent diabetes mellitus or IDDM) diabetes represents about 5 to
20 10% of this group and is the result of a progressive autoimmune destruction of the pancreatic β -cells with subsequent insulin deficiency. Type II (non-insulin dependent diabetes mellitus or NIDDM) diabetes represents 90-95% of the affected population but is much less well understood from the point of view of primary pathogenesis. Type II diabetic patients exhibit elements of both insulin resistance and relative insulin
25 deficiency. Diabetes often leads to further complications.

 Obesity represents the most prevalent of metabolic disorder, and it is the most important nutritional disorder in the western world, with estimates of its prevalence ranging from 30% to 50% within the middle-aged population. Obesity, also contributes to other diseases, such as an increased incidence of diseases such as coronary artery
30 disease, hypertension, stroke, diabetes, hyperlipidemia, and some cancers (See, *e.g.*, Nishina, P. M. *et al.*, 1994, *Metab.* 43: 554-558; Grundy, S. M. & Barnett, J. P., 1990, *Dis. Mon.* 36: 641-731).

Summary of the Invention

The present invention provides methods of treating metabolic disorders in a safe and effective manner where TNF α activity is detrimental. Excessive or unregulated TNF production has been implicated in mediating or exacerbating a number of diseases. People suffering from metabolic disorders, such as obesity and diabetes, have elevated levels of certain cytokines, including tumor necrosis factor α (TNF α), circulating in their blood (Spiegelman and Hotamisligil (1993) *Cell* 73:525; Chu *et al.* (2000) *Int J Obes Relat Metab Disord.* 24:1085; Ishii *et al.* (2000) *Metabolism.* 49:1616).

- 10 In one aspect, the invention is a subject comprising administering to the subject a therapeutically effective amount of a TNF α inhibitor such that the metabolic disorder is treated. In one embodiment, the TNF α antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF α with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of $1 \times 10^{-3} \text{ s}^{-1}$ or less, both determined by
- 15 surface plasmon resonance, and neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less.

In one embodiment, the invention provides a method of treating a subject suffering from metabolic disorder comprising administering a therapeutically effective amount a TNF α antibody, or an antigen-binding fragment thereof, wherein the

- 20 TNF α antibody dissociates from human TNF α with a K_{off} rate constant of $1 \times 10^{-3} \text{ s}^{-1}$ or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain
- 25 CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12. In one embodiment, the antibody or antigen-binding fragment thereof, is D2E7.

- 30 Furthermore, the invention provides a method of treating a subject suffering from a metabolic disorder, such as diabetes or obesity, comprising administering a

therapeutically effective amount a TNF α antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In one particular embodiment, the metabolic disorders is

5 diabetes or obesity. In one embodiment, the metabolic disorders treated is, for example, type 1 diabetes mellitus, type 2 diabetes mellitus, retinopathy, diabetic ulcerations, neuropathy, retinopathy ulcerations, peripheral neuropathy, diabetic macrovasculopathy, and obesity

In another embodiment, the invention provides a method of treating a subject

10 suffering from diabetes or obesity featuring administering a therapeutically effective amount of a TNF α antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF α with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s $^{-1}$ or less, both determined by surface plasmon resonance, and neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay

15 with an IC $_{50}$ of 1×10^{-7} M or less, such that said diabetes or obesity is treated.

A further embodiment of the invention provides a method of treating a subject suffering from diabetes or obesity featuring administering a therapeutically effective amount a TNF α antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNF α with a K_{off} rate constant of 1×10^{-3} s $^{-1}$ or less, as

20 determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4

25 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

In still another embodiment, the invention features a method of treating a subject suffering from diabetes or obesity comprising administering a therapeutically effective amount a TNF α antibody, or an antigen-binding fragment thereof, with a light chain

30 variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a

heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In one embodiment, the TNF α antibody, or antigen binding fragment thereof, is D2E7, also referred to as HUMIRA[®] (adalimumab). In one embodiment, the TNF α antibody is administered with at least one additional therapeutic agent.

- 5 The invention also provides a method for inhibiting human TNF α antibody activity in a human subject suffering from a metabolic disorder featuring administering a therapeutically effective amount of a TNF α antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF α with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s⁻¹ or less, both determined by
- 10 surface plasmon resonance, and neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay with an IC₅₀ of 1×10^{-7} M or less. In one embodiment the metabolic disorder is diabetes or obesity. In still a further embodiment, the metabolic disorder is, for example, type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic retinopathy, retinopathy ulcerations, neuropathy, diabetic ulcerations, peripheral neuropathy, and
- 15 diabetic macrovasculopathy. In another embodiment the TNF α antibody, or antigen-binding fragment thereof, is D2E7.

- The invention also describes a method for inhibiting human TNF α activity in a human subject suffering from diabetes or obesity, featuring administering a therapeutically effective amount of a TNF α antibody, or an antigen-binding fragment
- 20 thereof, to the subject, wherein the antibody dissociates from human TNF α with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s⁻¹ or less, both determined by surface plasmon resonance, and neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay with an IC₅₀ of 1×10^{-7} M or less. In one embodiment, the antibody, or antigen binding fragment thereof, is D2E7.

- 25 In yet another embodiment, the invention provides a method of treating a subject suffering from a metabolic disorder comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that the metabolic is treated. Also, the metabolic disorder is, for example, diabetes or obesity, type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic retinopathy, diabetic
- 30 ulcerations, retinopathy ulcerations, neuropathy, peripheral neuropathy, mellitus,

diabetic macrovasculopathy, and obesity. In yet another embodiment, D2E7 is administered in combination with or in the presence of additional therapeutic agent.

The invention also provides a method of treating a subject suffering from diabetes or obesity comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that said diabetes or obesity is treated.

In one embodiment, the invention features a method of treating a subject suffering from a metabolic disorder comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, and at least one additional therapeutic agent to the subject, such that the metabolic disorder is treated.

In yet another embodiment, the invention provides a kit comprising a pharmaceutical composition comprising a TNF α antibody, or an antigen binding portion thereof, and a pharmaceutically acceptable carrier; as well as instructions for administering to a subject the TNF α antibody pharmaceutical composition for treating a subject who is suffering from a metabolic disorder. In another embodiment, the TNF α antibody, or an antigen binding portion thereof, is D2E7. In another embodiment, the invention pertains to packaged pharmaceutical compositions featuring a TNF α inhibitor and instructions for using the inhibitor to treat a metabolic disorder, such as diabetes and obesity.

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Detailed Description of the Invention

This invention pertains to methods of treating metabolic disorders in which TNF α activity, e.g., human TNF α activity, is detrimental. The methods include administering to the subject an effective amount of a TNF α inhibitor, such that the metabolic disorder is treated. The invention also pertains to methods wherein the TNF α inhibitor is administered in combination with another therapeutic agent to treat a metabolic disorder. Various aspects of the invention relate to treatment with antibodies and antibody fragments, and pharmaceutical compositions comprising a TNF α inhibitor, and a pharmaceutically acceptable carrier for the treatment of a metabolic disorder.

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Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

- The term "human TNF α " (abbreviated herein as hTNF α , or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNF α is described further in, for example, Pennica, D. *et al.* (1984) *Nature* 312:724-729; Davis, J.M. *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y. *et al.* (1989) *Nature* 338:225-228. The term human TNF α is intended to include recombinant human TNF α (rhTNF α), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN). TNF α is also referred to as TNF.

- The term "TNF α inhibitor" includes agents which inhibit TNF α . Examples of TNF α inhibitors include etanercept (Enbrel[®], Amgen), infliximab (Remicade[®], Johnson and Johnson), human anti-TNF monoclonal antibody (D2E7/HUMIRA[®], Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech) and other compounds which inhibit TNF α activity, such that when administered to a subject suffering from or at risk of suffering from a disorder in which TNF α activity is detrimental, the disorder is treated. In one embodiment, a TNF α inhibitor is a compound, excluding etanercept and infliximab, which inhibits TNF α activity. In another embodiment, the TNF α inhibitors of the invention are used to treat a TNF α -related disorder, as described in more detail in section II. In one embodiment, the TNF α inhibitor, excluding etanercept and infliximab, is used to treat a TNF α -related disorder. In another embodiment, the TNF α inhibitor, excluding etanercept and infliximab, is used to treat a metabolic disorder. The term also includes each of the anti-TNF α human antibodies and antibody portions described herein as well as those described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356.

- The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant

region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The antibodies of the invention are described in further detail in U.S. Patent Nos. 6,090,382; 6,258,562; and 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, hTNF α). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL

domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J. *et al.* (1994) *Structure* 2:1121-1123). The antibody portions of the invention are described in further detail in U.S. Patent Nos. 6,090,382, 6,258,562, 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', F(ab')₂, Fabc, Fv, single chains, and single-chain antibodies. Other than "bispecific" or "bifunctional" immunoglobulins or antibodies, an immunoglobulin or antibody is understood to have each of its binding sites identical. A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny *et al.*, *J. Immunol.* 148, 1547-1553 (1992).

A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for

example in the CDRs and in particular CDR3. However, the term “human antibody”, as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

5 The term “recombinant human antibody”, as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated
10 from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D. *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline
15 immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not
20 naturally exist within the human antibody germline repertoire *in vivo*.

 An “isolated antibody”, as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNF α is substantially free of antibodies that specifically bind antigens other than hTNF α). An isolated antibody that specifically
25 binds hTNF α may, however, have cross-reactivity to other antigens, such as hTNF α molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

 A “neutralizing antibody”, as used herein (or an “antibody that neutralized hTNF α activity”), is intended to refer to an antibody whose binding to hTNF α results in
30 inhibition of the biological activity of hTNF α . This inhibition of the biological activity of hTNF α can be assessed by measuring one or more indicators of hTNF α biological activity, such as hTNF α -induced cytotoxicity (either *in vitro* or *in vivo*), hTNF α -induced

cellular activation and hTNF α binding to hTNF α receptors. These indicators of hTNF α biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see U.S. Patent No. 6,090,382). Preferably, the ability of an antibody to neutralize hTNF α activity is assessed by inhibition of hTNF α -induced

5 cytotoxicity of L929 cells. As an additional or alternative parameter of hTNF α activity, the ability of an antibody to inhibit hTNF α -induced expression of ELAM-1 on HUVEC, as a measure of hTNF α -induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by

10 detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnsson, B., *et al.* (1995) *J. Mol. Recognit.* 8:125-131; and Johnsson, B., *et al.* (1991) *Anal. Biochem.*

15 198:268-277.

The term " K_{off} ", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term " K_d ", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

20 The term " IC_{50} " as used herein, is intended to refer to the concentration of the inhibitor required to inhibit the biological endpoint of interest, e.g., neutralize cytotoxicity activity.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or

25 double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNF α , is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences

30 encoding antibodies or antibody portions that bind antigens other than hTNF α , which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for

example, an isolated nucleic acid of the invention encoding a VH region of an anti-hTNF α antibody contains no other sequences encoding other VH regions that bind antigens other than hTNF α .

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The term "dosing", as used herein, refers to the administration of a substance (*e.g.*, an anti-TNF α antibody) to achieve a therapeutic objective (*e.g.*, the treatment of a TNF α -associated disorder).

The terms “biweekly dosing regimen”, “biweekly dosing”, and “biweekly administration”, as used herein, refer to the time course of administering a substance (e.g., an anti-TNF α antibody) to a subject to achieve a therapeutic objective (e.g., the treatment of a TNF α -associated disorder). The biweekly dosing regimen is not intended to include a weekly dosing regimen. Preferably, the substance is administered every 9-19 days, more preferably, every 11-17 days, even more preferably, every 13-15 days, and most preferably, every 14 days.

The term “combination” as in the phrase “a first agent in combination with a second agent” includes co-administration of a first agent and a second agent, which for example may be dissolved or intermixed in the same pharmaceutically acceptable carrier, or administration of a first agent, followed by the second agent, or administration of the second agent, followed by the first agent. The present invention, therefore, includes methods of combination therapeutic treatment and combination pharmaceutical compositions.

The term “concomitant” as in the phrase “concomitant therapeutic treatment” includes administering an agent in the presence of a second agent. A concomitant therapeutic treatment method includes methods in which the first, second, third, or additional agents are co-administered. A concomitant therapeutic treatment method also includes methods in which the first or additional agents are administered in the presence of a second or additional agents, wherein the second or additional agents, for example, may have been previously administered. A concomitant therapeutic treatment method may be executed step-wise by different actors. For example, one actor may administer to a subject a first agent and a second actor may administer to the subject a second agent, and the administering steps may be executed at the same time, or nearly the same time, or at distant times, so long as the first agent (and additional agents) are administered in the presence of the second agent (and additional agents). The actor and the subject may be the same entity (e.g., human).

The term “combination therapy”, as used herein, refers to the administration of two or more therapeutic substances, e.g., an anti-TNF α antibody and another drug, such as a DMARD or NSAID. The other drug(s) may be administered concomitant with, prior to, or following the administration of an anti-TNF α antibody.

The term “metabolic disorder,” as used herein, refers to diseases or disorders which affect how the body processes substances needed to carry out physiological functions. Examples of metabolic disorders include, but are not limited to, diabetes and obesity. In one embodiment of the invention, the term “metabolic disorder” is used to refer to disorders which affect how the body processes substances needed to carry out physiological functions, excluding autoimmune diabetes.

The term “diabetes” or “diabetic disorder” or “diabetes mellitus,” as used interchangeably herein, refers to a disease which is marked by elevated levels of sugar (glucose) in the blood. Diabetes can be caused by too little insulin (a chemical produced by the pancreas to regulate blood sugar), resistance to insulin, or both.

The phrase “disorders associated with diabetes,” as used herein, refers to conditions and other diseases which are commonly associated with or related to diabetes. Example of disorders associated with diabetes include, for example, hyperglycemia, hyperinsulinaemia, hyperlipidaemia, insulin resistance, impaired glucose metabolism, obesity, diabetic retinopathy, macular degeneration, cataracts, diabetic nephropathy, glomerulosclerosis, diabetic neuropathy, erectile dysfunction, premenstrual syndrome, vascular restenosis, ulcerative colitis, coronary heart disease, hypertension, angina pectoris, myocardial infarction, stroke, skin and connective tissue disorders, foot ulcerations, metabolic acidosis, arthritis, and osteoporosis.

The term “obesity” as used herein, refers to a condition in which the subject has an excess of body fat relative to lean body mass. In one embodiment, obesity refers to a condition in which an individual weighs at least about 20% or more over the maximum desirable for their height. When an adult is more than 100 pounds overweight, he or she is considered to be “morbidly obese.” In another embodiment, obesity is defined as a BMI (body mass index) over 30 kg/m².

The term “kit” as used herein refers to a packaged product comprising components with which to administer the TNF α antibody of the invention for treatment of a TNF α -related disorder. The kit preferably comprises a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which are preferably contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can

also include instructions for administering the TNF α antibody of the invention.

Various aspects of the invention are described in further detail herein.

I. TNF α Inhibitors of the Invention

5 The invention provides methods of treating metabolic disorders in which the administration of a TNF α inhibitor is beneficial. In one embodiment, these methods include administration of isolated human antibodies, or antigen-binding portions thereof, that bind to human TNF α with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant,
10 neutralizing human anti-hTNF α antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 (the amino acid sequence of the D2E7 VL region is shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is shown in SEQ ID NO: 2). D2E7 is also referred to as HUMIRA[®] and aadalimumab. The properties of D2E7 have been described in Salfeld *et al.*, U.S.
15 patent No. 6,090,382, which is incorporated by reference herein.

 In one embodiment, the treatment of the invention includes the administration of D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNF α with low dissociation kinetics and high
20 neutralizing capacity. In one embodiment, the invention provides treatment with an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNF α with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s⁻¹ or less, both determined by surface plasmon resonance, and neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay with an IC₅₀ of 1×10^{-7} M or less. More
25 preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNF α with a K_{off} of 5×10^{-4} s⁻¹ or less, or even more preferably, with a K_{off} of 1×10^{-4} s⁻¹ or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNF α cytotoxicity in a standard *in vitro* L929 assay with an IC₅₀ of 1×10^{-8} M or less, even more preferably with an IC₅₀ of 1
30 $\times 10^{-9}$ M or less and still more preferably with an IC₅₀ of 1×10^{-10} M or less. In a

preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof.

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen.

- 5 Accordingly, in another aspect, the invention pertains to methods of treating inflammatory disorders in which the TNF α activity is detriment by administering human antibodies that have slow dissociation kinetics for association with hTNF α and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. Position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without
- 10 substantially affecting the K_{off} . Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off} . Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ
- 15 ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off} . Still further, the skilled
- 20 artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid
- 25 substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNF α . Positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNF α and thus, conservative amino acid substitutions preferably are not made at these positions
- 30 (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above) (*see* U.S. Patent No. 6,090,382).

Accordingly, in another embodiment, the invention provides methods of treating metabolic disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains the following characteristics:

- 5 a) dissociates from human TNF α with a K_{off} rate constant of $1 \times 10^{-3} \text{ s}^{-1}$ or less, as determined by surface plasmon resonance;
- b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;
- 10 c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.
- 15 More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF α with a K_{off} of $5 \times 10^{-4} \text{ s}^{-1}$ or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF α with a K_{off} of $1 \times 10^{-4} \text{ s}^{-1}$ or less.

- In yet another embodiment, the invention provides methods of treating metabolic disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR)
- 20 having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7
- 25 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1) and the HCVR has a

CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably are from the V_κ1 human germline family, more preferably from the A20 human germline V_κ gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B of
5 U.S. Patent No. 6,090,382. The framework regions for VH preferably are from the V_H3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B U.S. Patent No. 6,090,382.

Accordingly, in another embodiment, the invention provides methods of treating
10 metabolic disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain
15 embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably,
20 the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides methods of treating metabolic disorders in which the administration of an anti-TNF α antibody is beneficial administration of an isolated human antibody, or an antigen-binding portions thereof.
25 The antibody or antigen-binding portion thereof preferably contains D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16,
30 SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an

amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

- In another embodiment, the TNF α inhibitor of the invention is etanercept (described in WO 91/03553 and WO 09/406476), infliximab (described in U.S. Patent No. 5,656,272), CDP571 (a humanized monoclonal anti-TNF-alpha IgG4 antibody), CDP 870 (a humanized monoclonal anti-TNF-alpha antibody fragment), D2E7/HUMIRA[®] (a human anti-TNF mAb), soluble TNF receptor Type I, or a pegylated soluble TNF receptor Type I (PEGs TNF-R1).
- The TNF α antibody of the invention can be modified. In some embodiments, the TNF α antibody or antigen binding fragments thereof, is chemically modified to provide a desired effect. For example, pegylation of antibodies and antibody fragments of the invention may be carried out by any of the pegylation reactions known in the art, as described, for example, in the following references: *Focus on Growth Factors* 3:4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of the antibodies and antibody fragments of the invention is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-CIO) alkoxy- or aryloxy-polyethylene glycol.

- Methods for preparing pegylated antibodies and antibody fragments of the invention will generally comprise the steps of (a) reacting the antibody or antibody fragment with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby the antibody or antibody fragment becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

- Pegylated antibodies and antibody fragments may generally be used to treat metabolic disorders by administration of the TNF α antibodies and antibody fragments described herein. Generally the pegylated antibodies and antibody fragments have

increased half-life, as compared to the nonpegylated antibodies and antibody fragments. The pegylated antibodies and antibody fragments may be employed alone, together, or in combination with other pharmaceutical compositions.

- In yet another embodiment of the invention, TNF α antibodies or fragments thereof can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see *e.g.*, Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.
- An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (*e.g.*, another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNF α antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

- One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

- Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. An antibody
- 5 may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and
- 10 diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

- An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell.
- 15 To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant
- 20 DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene
- 25 Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

- To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy
- 30 and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services,

NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V_H Segments Reveals a Strong Bias in their Usage"

5 *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human
10 germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the V_κ1 family of
human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31
germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

15 Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to
20 identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes
should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are
25 incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA
30 techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or

VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA
5 fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A.,
10 *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab
15 fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The
20 sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region,
25 but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence (Gly4-Ser)₃, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible
30 linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty *et al.*, *Nature* (1990) 348:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It

will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr⁻ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been

reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNF α . The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNF α by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows

for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

Recombinant human antibodies of the invention in addition to D2E7 or an antigen binding portion thereof, or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*TM phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982. Methods of isolating human antibodies with high affinity and a low off rate constant for hTNF α are described in U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015, each of which is incorporated by reference herein.

II. Uses of TNF α Inhibitors of the Invention

The invention provides a method for inhibiting TNF α activity in a subject suffering from a metabolic disorder in which TNF α activity is detrimental. In one embodiment, the invention provides a method for inhibiting TNF α activity in a subject suffering from a metabolic disorder, including, for example, diabetes and obesity. In one
5 embodiment, the TNF α inhibitor is D2E7, also referred to as HUMIRA[®] (adalimumab).

TNF α has been implicated in the pathophysiology of a wide variety of disorders, including metabolic disorders, such as diabetes and obesity (Spiegelman and Hotamisligil (1993) *Cell* 73:625; Chu *et al.* (2000) *Int J Obes Relat Metab Disord.* 24:1085; Ishii *et al.* (2000) *Metabolism.* 49:1616). The invention provides methods for
10 TNF α activity in a subject suffering from such a metabolic disorder, which method comprises administering to the subject an antibody, antibody portion, or other TNF α inhibitor such that TNF α activity in the subject suffering from a metabolic disorder is inhibited. Preferably, the TNF α is human TNF α and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNF α with which an antibody
15 of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNF α (e.g., by administration of hTNF α or by expression of an hTNF α transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNF α with which
20 the antibody cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time courses of administration). Examples of animal models for evaluating the efficacy of a TNF α antibody for the treatment of a metabolic disorder
25 include NOD transgenic mice, Akita mice, NSY transgenic mice and *ob/ob* mice (see Baeder *et al.* (1992) *Clin Exp Immunol.* 89:174; Haseyama *et al.* (2002) *Tohoku J Exp Med.* 198:233; Makino *et al.* (1980): *Exp.Anim.* 29:1; Kolb (1987) *Diabetes/Metabolism Reviews* 3:751; Hamada *et al.* (2001) *Metabolism.* 50:1282; Coleman, (1978) *Diabetologia*, 14:141; Bailey *et al.* (1982) *Int.J.Obesity* 6:11).

30 As used herein, the term "a metabolic disorder in which TNF α activity is detrimental" is intended to include metabolic diseases and other disorders in which the

presence of TNF α in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder, including metabolic disorders, *e.g.*, diabetes and obesity. Accordingly, a metabolic disorder in which TNF α activity is

5 detrimental is a disorder in which inhibition of TNF α activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNF α in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNF α in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an

10 anti-TNF α antibody as described above. The use of the antibodies, antibody portions, and other TNF α inhibitors of the invention in the treatment of specific inflammatory disorders including metabolic disorders, as discussed further below. In certain embodiments, the antibody, antibody portion, or other TNF α inhibitor of the invention is administered to the subject in combination with another therapeutic agent for the

15 treatment of metabolic disorders, as described below in Section III.

The TNF α antibody of the invention can also be used to treat subjects who are at risk of developing a metabolic disorder. Metabolic disorders are often associated with arthritis, including rheumatoid arthritis. In one embodiment, the antibody of the invention is used to treat a subject who suffers from a metabolic disorder associated with

20 rheumatoid arthritis.

In another embodiment, the TNF α antibody of the invention is used to treat disorders associated with diabetes or obesity

Metabolic disorders affect how the body processes substances needed to carry out physiological functions. A number of metabolic disorders of the invention share certain characteristics, *i.e.* they are associated the insulin resistance, lack of ability to

25 regulate blood sugar, weight gain, and increase in body mass index. Examples of metabolic disorders include diabetes and obesity. Examples of diabetes include type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic neuropathy, peripheral neuropathy, diabetic retinopathy, diabetic ulcerations, retinopathy ulcerations, diabetic

30 macrovasculopathy, and obesity. Examples of metabolic disorders which can be treated with the TNF α antibody of the invention are described in more detail below:

A. *Diabetes*

Tumor necrosis factor has been implicated in the pathophysiology of diabetes. (see *e.g.*, Navarro J.F., Mora C., Maca, *Am J Kidney Dis.* 2003 Jul;42(1):53-61; Daimon M *et al.*, *Diabetes Care.* 2003 Jul;26(7):2015-20; Zhang M *et al.*, *J Tongji Med Univ.* 1999;19(3):203-5; Barbieri M *et al.*, *Am J Hypertens.* 2003 Jul;16(7):537-43.) For example, TNF α is implicated in the pathophysiology for insulin resistance. It has been found that serum TNF levels in patients with gastrointestinal cancer correlates with insulin resistance (see *e.g.*, McCall, J. *et al.* *Br. J. Surg.* 1992; 79: 1361-3).

Diabetes includes the two most common types of the disorder, namely type I diabetes and type II diabetes, which both result from the body's inability to regulate insulin. Insulin is a hormone released by the pancreas in response to increased levels of blood sugar (glucose) in the blood.

The term "type 1 diabetes," as used herein, refers to a chronic disease that occurs when the pancreas produces too little insulin to regulate blood sugar levels appropriately. Type 1 diabetes is also referred to as insulin-dependent diabetes mellitus, IDDM, juvenile onset diabetes, and diabetes - type I. Type 1 diabetes represents the result of a progressive autoimmune destruction of the pancreatic β -cells with subsequent insulin deficiency.

The term "type 2 diabetes," refers to a chronic disease that occurs when the pancreas does not make enough insulin to keep blood glucose levels normal, often because the body does not respond well to the insulin. Type 2 diabetes is also referred to as noninsulin-dependent diabetes mellitus, NDDM, and diabetes - type II

Diabetes can be diagnosed by the administration of a glucose tolerance test.

Clinically, diabetes is often divided into several basic categories. Primary examples of these categories include, autoimmune diabetes mellitus, non-insulin-dependent diabetes mellitus (type 1 NDDM), insulin-dependant diabetes mellitus (type 2 IDDM), non-autoimmune diabetes mellitus, non-insulin-dependant diabetes mellitus (type 2 NIDDM), and maturity-onset diabetes of the young (MODY). A further category, often referred to as secondary, refers to diabetes brought about by some identifiable condition which causes or allows a diabetic syndrome to develop. Examples of secondary categories include, diabetes caused by pancreatic disease, hormonal abnormalities, drug-

or chemical-induced diabetes, diabetes caused by insulin receptor abnormalities, diabetes associated with genetic syndromes, and diabetes of other causes. (see *e.g.*, Harrison's (1996) 14th ed., New York, McGraw-Hill).

Diabetes manifests itself in the foregoing categories and can cause several complications that are discussed in the following sections. Accordingly, the antibody, or antigen-binding fragment thereof, of the invention can be used to treat diabetes. In one embodiment, the TNF α antibody, or antigen-binding fragment thereof, of the invention is used to treat diabetes associated with the above identified categories.

Diabetes is often treated with diet, insulin dosages, and various medications described herein. Accordingly, the TNF α antibody of the invention may also be administered in combination with agents commonly used to treat metabolic disorders and pain commonly associated with diabetes.

In one embodiment, the TNF α antibody of the invention can also be used to treat disorders associated with diabetes. Diabetes manifests itself in many complications and conditions associated with diabetes, including the following categories:

i. *Diabetic Neuropathy and Peripheral Neuropathy*

Tumor necrosis factor has been implicated in the pathophysiology of diabetic neuropathy and peripheral neuropathy. (See Benjafeld *et al.* (2001) *Diabetes Care* 24:753; Qiang, X. *et al.* (1998) *Diabetologia* 41:1321-6; Pfeiffer *et al.* (1997) *Horm Metab Res.* 29:111).

The term "neuropathy," also referred to as nerve damage-diabetic, as used herein, refers to a common complication of diabetes in which nerves are damaged as a result of hyperglycemia (high blood sugar levels). A variety of diabetic neuropathies are recognized, such as distal sensorimotor polyneuropathy, focal motor neuropathy, and autonomic neuropathy.

The term "peripheral neuropathy," also known as peripheral neuritis and diabetic neuropathy, as used herein, refers to the failure of the nerves to carry information to and from the brain and spinal cord. Peripheral neuropathy produces symptoms such as pain, loss of sensation, and the inability to control muscles. In some cases, the failure of nerves to control blood vessels, intestinal function, and other organs results in abnormal blood pressure, digestion, and loss of other basic involuntary processes. Peripheral

neuropathy may involve damage to a single nerve or nerve group (mononeuropathy) or may affect multiple nerves (polyneuropathy).

Neuropathies that affect small myelinated and unmyelinated fibers of the sympathetic and parasympathetic nerves are known as “peripheral neuropathies.”

- 5 Furthermore, the related disorder of peripheral neuropathy, also known as peripheral neuritis and diabetic neuropathy, refers to the failure of the nerves to carry information to and from the brain and spinal cord. This produces symptoms such as pain, loss of sensation, and the inability to control muscles. In some cases, failure of nerves controlling blood vessels, intestinal function, and other organs results in abnormal blood pressure, digestion, and loss of other basic involuntary processes. Peripheral neuropathy
10 may involve damage to a single nerve or nerve group (mononeuropathy) or may affect multiple nerves (polyneuropathy).

The term “diabetic neuropathy” refers to a common complication of diabetes in which nerves are damaged as a result of hyperglycemia (high blood sugar levels).

- 15 Diabetic neuropathy is also referred to as neuropathy and nerve damage-diabetic. A variety of diabetic neuropathies are recognized, such as distal sensorimotor polyneuropathy, focal motor neuropathy, and autonomic neuropathy.

(ii) Diabetic Retinopathy

- 20 Tumor necrosis factor has been implicated in the pathophysiology of diabetic retinopathy (Scholz *et al.* (2003) *Trends Microbiol.* 11:171). The term “diabetic retinopathy” as used herein, refers to progressive damage to the eye's retina caused by long-term diabetes. Diabetic retinopathy, includes proliferative retinopathy. Proliferative neuropathy in turn includes includes neovascularization, peritinal
25 hemorrhage and retinal detachment.

- In advanced retinopathy, small vessels proliferate on the surface of the retina. These blood vessels are fragile, tend to bleed and can cause peritinal hemorrhages. The hemorrhage can obscure vision, and as the hemorrhage is resorbed fibrous tissue forms predisposing to retinal detachments and loss of vision. In addition, diabetic retinopathy
30 includes proliferative retinopathy which includes neovascularization, peritinal hemorrhage and retinal detachment. Daibetic retinopathy also includes “background retinopathy” which involves changes occuring with the layers of the retina.

(iii) *Diabetic Ulcerations and Retinopathy Ulcerations*

Tumor necrosis factor has been implicated in the pathophysiology of diabetic ulcerations, (see Lee *et al.* (2003) *Hum Immunol.* 64:614; Navarro *et al.* (2003) *Am J Kidney Dis.* 42:53; Daimon *et al.* (2003) *Diabetes Care.* 26:2015; Zhang *et al.* (1999) *J Tongji Med Univ.* 19:203; Barbieri *et al.* (2003) *Am J Hypertens.* 16:537; Venn *et al.* (1993) *Arthritis Rheum.* 36:819; Westacott *et al.* (1994) *J Rheumatol.* 21:1710).

The term “diabetic ulcerations,” as used herein, refers to an ulcer which results as a complication of diabetes. An ulcer is a crater-like lesion on the skin or mucous membrane caused by an inflammatory, infectious, malignant condition, or metabolic disorder. Typically diabetic ulcers can be found on limbs and extremities, more typically the feet. These ulcers, caused by diabetic conditions, such as neuropathy and a vascular insufficiency, can lead to ischemia and poor wound healing. More extensive ulcerations may progress to osteomyelitis. Once osteomyelitis develops, it may be difficult to eradicate with antibiotics and amputation may be necessary..

The term “retinopathy ulcerations,” as used herein refers to an ulcer which causes or results in damages to the eye and the eye’s retina. Retinopathy ulcerations may include conditions such as retinopathic hemorrhages.

(iv) *Diabetic Macrovasculopathy*

Tumor necrosis factor has been implicated in the pathophysiology of diabetic macrovasculopathy (Devaraj *et al.* (2000) *Circulation.* 102:191; Hattori Y *et al.* (2000) *Cardiovasc Res.* 46:188; Clausell N *et al.* (1999) *Cardiovasc Pathol.* 8:145). The term “diabetic macrovasculopathy,” also referred to as “macrovascular disease,” as used herein, refers to a disease of the blood vessels that results from diabetes. Diabetic macrovasculopathy complication occurs when, for example, fat and blood clots build up in the large blood vessels and stick to the vessel walls. Diabetic macrovasculopathies include diseases such as coronary disease, cerebrovascular disease, and peripheral vascular disease, hyperglycaemia and cardiovascular disease, and strokes.

B. *Obesity*

Tumor necrosis factor has been implicated in the pathophysiology of obesity (see e.g., Pihlajamaki J *et al.* (2003) *Obes Res.* 11:912; Barbieri *et al.* (2003) *Am J Hypertens.* 16:537; Tsuda *et al.* (2003) *J Nutr.* 133:2125). Obesity increases a person's risk of illness and death due to diabetes, stroke, coronary artery disease, hypertension, high cholesterol, and kidney and gallbladder disorders. Obesity may also increase the risk for some types of cancer, and may be a risk factor for the development of osteoarthritis and sleep apnea. Obesity can be treated with the antibody of the invention alone or in combination with other metabolic disorders, including diabetes.

It is understood that all of the above-mentioned disorders include both the adult and juvenile forms of the disease where appropriate. It is also understood that all of the above-mentioned disorders include both chronic and acute forms of the disease wherein appropriate. In addition, the TNF α antibody of the invention can be used to treat each of the above-mentioned TNF α -related disorders alone or in combination with one another, e.g., a subject who is suffering from obesity and diabetes.

III. Pharmaceutical Compositions and Pharmaceutical Administration

A. *Compositions and Administration*

The antibodies, antibody-portions, and other TNF α inhibitors of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject for the treatment of a metabolic disorder. Typically, the pharmaceutical composition comprises an antibody, antibody portion, or other TNF α inhibitor of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or

emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody, antibody portion, or other TNF α inhibitor.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies or other TNF α inhibitors. The preferred mode of administration is parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody or other TNF α inhibitor is administered by intravenous infusion or injection. In another preferred embodiment, the antibody or other TNF α inhibitor is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i.e., antibody, antibody portion, or other TNF α inhibitor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents. For example, an anti-hTNF α antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more DMARD or one or more NSAID or one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNF α receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNF α production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751) or any combination thereof. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

In one embodiment, the invention includes pharmaceutical compositions comprising an effective amount of a TNF α inhibitor and a pharmaceutically-acceptable carrier, wherein the effective amount of the TNF α inhibitor may be effective to treat a metabolic disease, including, for example, type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic retinopathy, diabetic ulcerations, neuropathy, retinopathy ulcerations, peripheral neuropathy, diabetic macrovasculopathy, and obesity

The antibodies, antibody-portions, and other TNF α inhibitors of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the

art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

The TNF α antibodies of the invention can also be administered in the form of protein crystal formulations which include a combination of protein crystals encapsulated within a polymeric carrier to form coated particles. The coated particles of the protein crystal formulation may have a spherical morphology and be microspheres of up to 500 micro meters in diameter or they may have some other morphology and be microparticulates. The enhanced concentration of protein crystals allows the antibody of the invention to be delivered subcutaneously. In one embodiment, the TNF α antibodies of the invention are delivered via a protein delivery system, wherein one or more of a protein crystal formulation or composition, is administered to a subject with a TNF α -related disorder. Compositions and methods of preparing stabilized formulations of whole antibody crystals or antibody fragment crystals are also described in WO 02/072636, which is incorporated by reference herein. In one embodiment, a formulation comprising the crystallized antibody fragments described in Examples 5 and 6 are used to treat a TNF α -related disorder.

In certain embodiments, an antibody, antibody portion, or other TNF α inhibitor of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody, antibody portion, or other TNF α inhibitor may vary according to factors such as the disease state, age, sex, and

weight of the individual, and the ability of the antibody, antibody portion, other TNF α inhibitor to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, antibody portion, or other TNF α inhibitor are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 10-150 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the metabolic condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Ranges intermediate to the above recited concentrations, *e.g.*, about 6-144 mg/ml, are also intended to be part

of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The invention also pertains to packaged pharmaceutical compositions or kits which comprise a TNF α inhibitor of the invention and instructions for using the inhibitor to treat metabolic disorders, including diabetes and obesity, as described above. In one embodiment, the kit comprises a single pharmaceutical composition comprising an anti-TNF α antibody, one or more drugs useful for treating a metabolic disorder and a pharmaceutically acceptable carrier. The kit contains instructions for dosing of the pharmaceutical compositions for the treatment of a disorder in which the administration of an anti-TNF α antibody is beneficial, such as a metabolic disorder, especially diabetes and/or obesity. The package or kit alternatively can contain the TNF α inhibitor and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged pharmaceuticals or kits further can include a second agent (as described herein) packaged with or copromoted with instructions for using the second agent with a first agent (as described herein).

B. *Additional therapeutic agents*

The invention pertains to pharmaceutical compositions and methods of use thereof for the treatment of metabolic disorder, including diabetes and obesity. The pharmaceutical compositions comprise a first agent that prevents or inhibits metabolic disorders. The pharmaceutical composition also may comprise a second agent that is an active pharmaceutical ingredient; that is, the second agent is therapeutic and its function is beyond that of an inactive ingredient, such as a pharmaceutical carrier, preservative, diluent, or buffer. The second agent may be useful in treating or preventing metabolic disorders. The second agent may diminish or treat at least one symptom(s) associated with the targeted disease. The first and second agents may exert their biological effects by similar or unrelated mechanisms of action; or either one or both of the first and second agents may exert their biological effects by a multiplicity of mechanisms of action. A pharmaceutical composition may also comprise a third compound, or even more yet, wherein the third (and fourth, *etc.*) compound has the same characteristics of a second agent.

It should be understood that the pharmaceutical compositions described herein may have the first and second, third, or additional agents in the same pharmaceutically acceptable carrier or in a different pharmaceutically acceptable carrier for each described embodiment. It further should be understood that the first, second, third and additional agent may be administered simultaneously or sequentially within described embodiments. Alternatively, a first and second agent may be administered simultaneously, and a third or additional agent may be administered before or after the first two agents.

The combination of agents used within the methods and pharmaceutical compositions described herein may have a therapeutic additive or synergistic effect on the condition(s) or disease(s) targeted for treatment. The combination of agents used within the methods or pharmaceutical compositions described herein also may reduce a detrimental effect associated with at least one of the agents when administered alone or without the other agent(s) of the particular pharmaceutical composition. For example, the toxicity of side effects of one agent may be attenuated by another agent of the composition, thus allowing a higher dosage, improving patient compliance, and improving therapeutic outcome. The additive or synergistic effects, benefits, and advantages of the compositions apply to classes of therapeutic agents, either structural or functional classes, or to individual compounds themselves.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating inflammatory disorders in which TNF α activity is detrimental, including metabolic disorders. For example, an anti-hTNF α antibody, antibody portion, or other TNF α inhibitor of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNF α receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNF α production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies or other TNF α inhibitors of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such

combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies. Specific therapeutic agent(s) are generally selected based on the particular disorder being treated, as discussed below.

- 5 Nonlimiting examples of therapeutic agents with which an antibody, antibody portion, or other TNF α inhibitor of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNF α antibody; Celltech/Bayer); cA2/infliximab (chimeric anti-TNF α antibody; Centocor); 75
- 10 kD TNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kD TNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see e.g., *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-
- 15 2 (IL-2 fusion proteins; Seragen; see e.g., *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2R α ; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Aingen); TNF-bp/s-TNF
- 20 (soluble TNF binding protein; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see
- 25 e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (e.g., Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor
- 30 of plasminogen activation; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see e.g., *Arthritis & Rheumatism*

- (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1 β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-converase inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin;
- 20 anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline;
- 25 anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca *et al.* (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); azaribine; methotrexate;
- 30 antivirals; and immune modulating agents. Any of the above-mentioned agents can be administered in combination with the TNF α antibody of the invention to treat a metabolic disease, including, for example, diabetes or obesity. Examples of diabetes

include, but are not limited to type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic retinopathy, diabetic ulcerations, neuropathy, retinopathy ulcerations, peripheral neuropathy, and diabetic macrovasculopathy.

- In one embodiment, the TNF α antibody of the invention is administered in
- 5 combination with one of the following agents for the treatment of rheumatoid arthritis: methotrexate, prednisone, celecoxib, folic acid, hydroxychloroquine sulfate, rofecoxib, etanercept, infliximab, leflunomide, naproxen, valdecoxib, sulfasalazine, methylprednisolone, ibuprofen, meloxicam, methylprednisolone acetate, gold sodium thiomalate, aspirin, azathioprine, triamcinolone acetoneide, propoxyphene napsylate/apap,
- 10 folate, nabumetone, diclofenac, piroxicam, etodolac, diclofenac sodium, oxaprozin, oxycodone hcl, hydrocodone bitartrate/apap, diclofenac sodium/misoprostol, fentanyl, anakinra, human recombinant, tramadol hcl, salsalate, sulindac, cyanocobalamin/fa/pyridoxine, acetaminophen, alendronate sodium, prednisolone, morphine sulfate, lidocaine hydrochloride, indomethacin, glucosamine
- 15 sulfate/chondroitin, cyclosporine, amitriptyline hcl, sulfadiazine, oxycodone hcl/acetaminophen, olopatadine hcl, misoprostol, naproxen sodium, omeprazole, mycophenolate mofetil, cyclophosphamide, rituximab, IL-1 TRAP, MRA, CTLA4-IG, IL-18 BP, ABT-874, ABT-325 (anti-IL 18), anti-IL 15, BIRB-796, SCIO-469, VX-702, AMG-548, VX-740, Roflumilast, IC-485, CDC-801, and mesopram. In another
- 20 embodiment, the TNF α antibody of the invention is administered for the treatment of a metabolic disorder in combination with one of the above mentioned agents for the treatment of rheumatoid arthritis.

- In one embodiment, the TNF α antibody of the invention is administered in combination with one of the following agents for the treatment of a metabolic disorder
- 25 in which TNF α activity is detrimental: anti-IL12 antibody (ABT 874); anti-IL18 antibody (ABT 325); small molecule inhibitor of LCK; small molecule inhibitor of COT; anti-IL1 antibody; small molecule inhibitor of MK2; anti-CD19 antibody; small molecule inhibitor of CXCR3; small molecule inhibitor of CCR5; small molecule inhibitor of CCR11 anti-E/L selectin antibody; small molecule inhibitor of P2X7; small
- 30 molecule inhibitor of IRAK-4; small molecule agonist of glucocorticoid receptor; anti-C5a receptor antibody; small molecule inhibitor of C5a receptor; anti-CD32 antibody; and CD32 as a therapeutic protein.

In yet another embodiment, the TNF α antibody of the invention is administered in combination with an antibiotic or antiinfective agent. Antiinfective agents include those agents known in the art to treat viral, fungal, parasitic or bacterial infections. The term, "antibiotic," as used herein, refers to a chemical substance that inhibits the growth of, or kills, microorganisms. Encompassed by this term are antibiotic produced by a microorganism, as well as synthetic antibiotics (e.g., analogs) known in the art. Antibiotics include, but are not limited to, clarithromycin (Biaxin[®]), ciprofloxacin (Cipro[®]), and metronidazole (Flagyl[®]).

Any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from a metabolic disorder in which TNF α is detrimental in combination with the TNF α antibody of the invention. In one embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to a TNF α antibody to treat a metabolic disease, including diabetes and obesity.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example 1: TNF α Inhibitor in Mouse Model for Diabetes

Study of TNF antibody in NOD mouse model

The following study is performed using the nonobese diabetic (NOD) mouse model for type 1 diabetes. At the onset of the study, insulin levels are established by testing glucose levels in the blood of the NOD mice. Baseline insulin levels are established by fasting the mice overnight (17 hours). The blood glucose level is checked, and checked again 4 minutes after administering glucose. Blood glucose is determined with a reflectance meter. Glucose (200 mg/mL in 0.85% sodium chloride) in 1 mL syringes were prewarmed to 40°C and mice injected ip at 3 g/kg body weight. The

second blood glucose measurement is determined 4 minutes after administering the glucose. Samples of the second blood measurement are used to determine the blood glucose level using the Glucometer Elite. The remaining sample of blood is collected into microfuge tube and used to separate the serum for insulin or C-peptide

- 5 determination. Insulin levels are determined using a rodent radioimmunoassay (RIA) kit per manufacturers' instruction or an enzyme-linked immunoassay (ELISA).

- Diabetic mice are chosen based on the criteria that they have blood glucose readings greater than 300 mg/dL. Non-diabetic mice are chosen such that their glucose readings are under 200 mg/dL by glucose meter. NOD mice (those which displayed the
- 10 glucose reading described above) are allowed to develop diabetes, and are administered doses of a placebo or a monoclonal anti-TNF α antibody which is known to bind and neutralize mouse TNF α , e.g., antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen). The mice receive daily subcutaneous injections of the TNF antibody or a
- 15 placebo. Insulin and glucose levels are measured at weekly increments to determine whether there is a decrease in blood glucose levels.

Example 2: TNF α Inhibitor in Mouse Model of Diabetes

- 20 *Study of TNF antibody in type-2 diabetic mouse model*

- The following study is performed using the NSY mouse model (type 2 diabetes) (Ueda *et al.* Diabetes Vol. 48, May 1999, 1168: 1174). The NSY mouse closely mimics human type 2 diabetes in that the onset is age-dependant, the animals are not severely
- 25 obese, and both insulin resistance and impaired insulin response to glucose contribute to disease development. This study evaluates a number of phenotypic data, including glucose levels, insulin levels, height, and weight of the mouse.

- Glucose is measured in the NSY mouse according to standard techniques, including by an intravenous glucose-tolerance test. Baseline glucose resistance is
- 30 measured prior to 12 weeks before the initiation of the study, and glucose, insulin, height, and weights are charted accordingly.

NSY mice are administered doses of either a placebo or a monoclonal anti-TNF α antibody which is known to bind and neutralize mouse TNF α , *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen). Mice receive daily subcutaneous injections of the anti-TNF antibody or a placebo. Glucose level measurements are taken 120 minutes after intraperitoneal glucose administration at 0, 12, 24, 36, and 48 weeks following the initiation of the study to examine whether there is a decrease in glucose intolerance.

10 **Example 3: TNF α Inhibitor in Obese Mouse Model**

Study of TNF antibody in mouse model for obesity

The following study is performed using the obese mice (*ob/ob*) murine model.

15 Mice are evaluated for weight loss and a reduction in their body mass index. Obese mice are characterized by marked obesity, hyperphagia, transient hyperglycemia and markedly elevated plasma insulin concentration associated with an increase in number and size of the beta cells of the islets of Langerhans (Coleman, *supra*). Obese mice (*ob/ob*) are phenotypically distinguished from their lean littermates (*ob/+* and *+/+*) at about 26 days of age on basis of body weight. Obese mice gain weight rapidly and have marked obesity at 5 weeks of age. Obese mice reach a maximum body weight of 60-70 grams at an age of 7-8 months, while lean littermates reach their maximal weight of 30-40 grams in 3-4 months (Coleman, *supra*; Westman (1968) *Diabetologia* 4:141; Bray & York (1971) *Physiological reviews.* 51:598).

25 Thirteen (13) week old *ob/ob* mice and matched wild-type control mice are weighed to establish a base line weight. The mice are administered doses of either a placebo or a monoclonal anti-TNF α antibody which is known to bind and neutralize mouse TNF α , *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen).

30 Mice receive daily subcutaneous injections of the TNF antibody or a placebo. All mice are fed a high-fat diet (58% fat, Research Diets D12330) for 12 weeks. Body weights are recorded weekly. After 12 weeks, the mice are euthanized,

and the fat pads are dissected and weighed, as well as the final weight of the animal to determine the final body mass index (BMI) and occurrence of obesity.

Alternatively, *ob/ob* mice can be treated with D2E7 beginning at birth, and fed a regular diet, *i.e.*, not low-fat, not high-fat diet. Treated and control mice (*ob/ob* littermates) are weighed weekly. Normally, at five weeks *ob/ob* mice exhibit a BMI which indicates that they are obese. Mice are examined at five weeks to determine if they have a lower BMI measurement than the controls.

Example 4: TNF α Inhibitor in Treating Type 2 Diabetes in Humans

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Study of D2E7 in human subjects with diabetes type 2

Patients who are diagnosed with type 2 diabetic are selected for the study. The following inclusion criteria are used: 40-65 years of age, known duration of diabetes > 12 months, stable BMI < 35 kg/m², supine blood pressure < 140/90 mm/Hg, serum creatinine < 106 μ mol/l, m24-h UAE between 20 and 200 μ g/min in samples assessed weekly during the 3 months prior to the first evaluation and in the 15-day placebo run-in period, and no cardiovascular, hepatic, or systemic disease before the beginning of the study. The subjects do not take any additional drugs other than those for the treatment of their diabetes. For three days prior to and throughout the duration of the study, the patients follow an isocaloric diet (~0.13 mJ x kg⁻¹ X day⁻¹ ; 50% carbohydrates, 35% lipids, 15% proteins) with no restriction on sodium intake. Adherences to the dietary recommendations are checked at each visit.

Patients are administered 40 mg of D2E7 in a biweekly dosing regiment, although this dose and the frequency of the dose can be adjusted by an ordinarily skilled artisan with knowledge of HCV treatments. Patients are monitored at least every week for twelve weeks, with repeated assays like those which were performed prior to the initiation of the D2E7 treatment and as described below.

For each patient's evaluation throughout the study, the following baseline examinations are performed: supine blood pressure measurements; BMI; the mean of three twenty four hour urine samples; blood glucose levels; twenty four hour urine glucose; serum creatine levels; creatinine clearance; and an electrocardiogram reading.

Furthermore, each subject keeps a daily journal to monitor typical type 2 diabetic symptoms such as fatigue, excessive thirst, frequent urination, blurred vision, a high rate of infections, wounds that heal slowly, mood changes, and sexual problems. Patients are examined to determine if there is a reduction in blood glucose levels in D2E7, as well as reduction in symptoms typical to type II diabetes such as fatigue, excessive thirst, frequent urination, blurred vision, a high rate of infections, wounds that heal slowly, and mood changes.

Example 5: Crystallization of D2E7 F(ab)'₂ fragment

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Generation and purification of the D2E7 F(ab)'₂ Fragment

A D2E7 F(ab)'₂ fragment was generated and purified according to the following procedure. Two ml of D2E7 IgG (approximately 63 mg/ml) was dialyzed against 1 liter of Buffer A (20 mM NaOAc, pH 4) overnight. After dialysis, the protein was diluted to a concentration of 20 mg/ml. Immobilized pepsin (Pierce; 6.7 ml of slurry) was mixed with 27 ml of Buffer A, mixed, and centrifuged (Beckman floor centrifuge, 5000 rpm, 10 min). The supernatant was removed, and this washing procedure was repeated twice more. The washed immobilized pepsin was re-suspended in 13.3 ml of Buffer A. D2E7 (7.275 ml, 20 mg/ml, 145.5 mg) was mixed with 7.725 ml of Buffer A and 7.5 ml of the washed immobilized pepsin slurry. The D2E7/pepsin mixture was incubated at 37 °C for 4.5 hr with shaking (300 rpm). The immobilized pepsin was then separated by centrifugation. Analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was essentially complete (~115 kDa band unreduced, ~30 and ~32 kDa bands reduced).

The D2E7 F(ab)'₂ fragment was separated from intact D2E7 and Fc fragments using Protein A chromatography. One-half of the above reaction supernatant (10 ml) was diluted with 10 ml of Buffer B (20 mM Na phosphate, pH 7), filtered through a 0.45 µm Acrodisc filter, and loaded onto a 5 ml Protein A Sepharose column (Pharmacia Hi-Trap; previously washed with 50 ml of Buffer B). Fractions were collected. After the protein mixture was loaded, the column was washed with Buffer B until the absorbance at 280 nm re-established a baseline. Bound proteins were eluted with 5 ml of Buffer C (100 mM citric acid, pH 3); these fractions were neutralized by adding 0.2 ml of 2 M

Tris•HCl, pH 8.9. Fractions were analyzed by SDS-PAGE; those that contained the D2E7 F(ab)₂ fragment were pooled (~42 ml). Protein concentrations were determined by absorbance at 280 nm in 6 M guanidine•HCl, pH 7 (calculated extinction coefficients: D2E7, 1.39 (AU-ml)/mg; F(ab)₂, 1.36 (AU-ml)/mg). The flow-through pool contained ~38.2 mg protein (concentration, 0.91 mg/ml), which represents a 79% yield of F(ab)₂ (theoretical yield is ~2/3 of starting material, divided by two [only half purified], i.e. ~48.5 mg).

The D2E7 F(ab)₂ fragment was further purified by size-exclusion chromatography. The pooled Protein A flow-through was concentrated from ~42 to ~20 ml, and a portion (5 ml, ~7.5 mg) was then chromatographed on a Superdex 200 column (26/60, Pharmacia) previously equilibrated (and eluted) with Buffer D (20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA). Two peaks were noted by absorbance at 280 nm: Peak 1, eluting at 172–200 ml, consisted of F(ab)₂ (analysis by SDS-PAGE; ~115 kDa band unreduced, ~30 and ~32 kDa bands reduced); Peak 2, eluting at 236–248 ml, consisted of low molecular weight fragment(s) (~15 kDa, reduced or unreduced). Peak 1 was concentrated to 5.3 mg/ml for crystallization trials.

Crystallization of the D2E7 F(ab)₂ Fragment

The D2E7 F(ab)₂ fragment (5.3 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method by mixing equal volumes of F(ab)₂ and crystallization buffer (approx. 1 μ l of each) and allowing the mixture to equilibrate against the crystallization Buffer Bt 4 or 18 °C. The crystallization buffers used consisted of the Hampton Research Crystal Screens I (solutions 1–48) and II (solutions 1–48), Emerald Biostructures Wizard Screens I and II (each solutions 1–48), and the Jena Biosciences screens 1–10 (each solutions 1–24). Crystals were obtained under many different conditions, as summarized in Table 1.

Table 1. Summary of crystallization conditions for the D2E7 F(ab)₂ fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton I	32	4	2.0 M (NH ₄) ₂ SO ₄	tiny needle clusters
Hampton I	46	4	0.2 M Ca(Oac) ₂ , 0.1 M Na cacodylate pH 6.5, 18% PEG 8K	medium sized needle clusters
Hampton I	48	4	0.1 M Tris HCl pH 8.5, 2.0 M NH ₄ H ₂ PO ₄	micro needle clusters

Screen	Solution	Temp °C	Condition	Result
Hampton 2	2	4	0.01 M hexadecyltrimethylammonium bromide, 0.5 M NaCl, 0.01 M MgCl ₂	small shard crystals
Hampton 2	13	4	0.2 M (NH ₄) ₂ SO ₄ , 0.1 M NaOAc pH 4.6, 30% PEG MME 2000	small needle clusters
Hampton 2	15	4	0.5 M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 5.6, 1.0M Li ₂ SO ₄	large needle clusters
Hampton 2	16	4	0.5M NaCl, 0.1M NaOAc pH 5.6, 4% Ethylene Imine polymer	large irregular crystal
Hampton 1	34	18	0.1 NaOAc pH 4.6, 2.0 M Na Formate	needle clusters
Hampton 1	35	18	0.1M Hepes pH 7.5, 0.8M mono-sodium dihydrogen phosphate, 0.8M mono-potassium dihydrogen phosphate	needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2.0M NaCl	dense needle clusters
Hampton 2	12	18	0.1M CdCl ₂ , 0.1M NaOAc pH 4.6, 30% PEG 400	needles & amorphous crystals
Hampton 2	15	18	0.5M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 5.6, 1.0M Li ₂ SO ₄	tiny needle clusters
Wizard I	27	4	1.2M NaH ₂ PO ₄ , 0.8M K ₂ HPO ₄ , 0.1M CAPS pH 10.5, 0.2 M Li ₂ SO ₄	Medium large needle clusters
Wizard I	30	4	1.26M (NH ₄) ₂ SO ₄ , 0.1 M NaOAc pH 4.5, 0.2M NaCl	small needle clusters
Wizard II	8	4	10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl	Large plate crystals grown in clusters
Wizard II	43	4	10% PEK 8K, 0.1M Tris pH 7.0, 0.2 M MgCl ₂	micro needle clusters
Wizard I	4	18	35% MPD, 0.1M Imidazole pH 8.0, 0.2M MgCl ₂	rod-shaped crystal
Wizard I	27	18	1.2M NaH ₂ PO ₄ , 0.8M K ₂ HPO ₄ , 0.1M CAPS pH 10.5, 0.2 M Li ₂ SO ₄	Needle clusters
Wizard II	7	18	30% PEG 3K, 0.1M Tris pH 8.5, 0.2M NaCl	tiny needle clusters
Wizard II	11	18	10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac) ₂	tiny hexagonal or rhombohedral crystals
Wizard II	46	18	1.0M AP, 0.1M Imidazole pH 8.0, 0.2M NaCl	1 irregular crystal
JB 1	D6	4	30% PEG 3K, 0.1M Tris HCl pH 8.5, 0.2M Li ₂ SO ₄	tiny needles in precipitate
JB 2	B6	4	20% PEG 4K, 0.1M Tris HCl pH 8.5, 0.2M Na Cacodylate	tiny needle cluster balls
JB 3	A1	4	8% PEG 4K, 0.8M LiCl, 0.1M Tris HCl pH 8.5	Large frost-like crystals
JB 3	B1	4	15% PEG 4K, 0.2M (NH ₄) ₂ SO ₄	tiny needle clusters
JB 3	D5	4	30% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH ₄ OAc	tiny needles in precipitate.
JB 4	B1	4	15% PEG 6K, 0.05M KCl, 0.01M MgCl ₂	needle cluster balls
JB 3	A6	18	12% PEG 4K, 0.1M NaOAc pH 4.6, 0.2M NH ₄ OAc	needle clusters
JB 3	B1	18	15% PEG 4K, 0.2M (NH ₄) ₂ SO ₄	needle clusters in precipitate
JB 3	C6	18	25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH ₄ OAc	long, thin needles
JB 4	C5	18	8% PEG 8K, 0.2 M LiCl, 0.05M MgSO ₄	frost-like crystals
JB 5	A3	4	15% PEG 8K, 0.2M (NH ₄) ₂ SO ₄	long single needles in phase separation
JB 5	A4	4	15% PEG 8K, 0.5M Li ₂ SO ₄	tiny needle clusters
JB 5	A5	4	15% PEG 8K, 0.1M Na MES pH 6.5, 0.2M Ca(OAc) ₂	needle cluster balls
JB 6	B2	4	1.6M (NH ₄) ₂ SO ₄ , 0.5 LiCl	tiny needle cluster balls
JB 6	C2	4	2.0 M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 4.6	micro needle clusters

Screen	Solution	Temp °C	Condition	Result
JB 10	D3	18	2.0M Na Formate, 0.1M NaOAc pH 4.6	needle clusters

The following conditions (as described in Table 1) produced crystals which can be used for diffraction quality crystals: Wizard II, 11, 18, 10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)₂, tiny hexagonal or rhom. Xtals; Wizard II, 10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl, large plate xtals grown in clusters; JB 3, C6, 18, 25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M Ammonium Acetate, long, thin needles; Hampton 2, 15, 18, 0.5M AS, 0.1M Na Acetate trihydrate pH 5.6, 1.0M Li Sulfate monohydrate, tiny needle clusters.

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Example 6: Crystallization of D2E7 Fab fragment

Generation and purification of the D2E7 Fab Fragment

A D2E7 Fab fragment was generated and purified according to the following procedure. Four ml of D2E7 IgG (diluted to about 20 mg/ml) was diluted with 4 ml of Buffer E (20 mM Na phosphate, 5 mM cysteine•HCl, 10 mM EDTA, pH7) and mixed with 6.5 ml of a slurry of immobilized papain (Pierce, 1%; previously washed twice with 26 ml of Buffer E). The D2E7/papain mixture was incubated at 37 °C overnight with shaking (300 rpm). The immobilized papain and precipitated protein were separated by centrifugation; analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was partially complete (~55, 50, 34, and 30 kDa bands unreduced, with some intact and partially digested D2E7 at ~115 and ~150 kDa; ~30 and ~32 kDa bands reduced, as well as a ~50 kDa band). Nonetheless, the digestion was halted and subjected to purification.

The D2E7 Fab fragment was purified by Protein A chromatography and Superdex 200 size-exclusion chromatography essentially as described above for the F(ab)₂ fragment. The Protein A column flow-through pool (21 ml) contained ~9.2 mg (0.44 mg/ml), whereas the Protein A eluate (4 ml) contained ~19.5 mg (4.9 mg/ml). Analysis by SDS-PAGE indicated that the flow-through was essentially pure Fab fragment (~48 and ~30 kDa unreduced, broad band at ~30 kDa reduced), whereas the

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eluate was intact and partially-digested D2E7. The Fab fragment was further purified on a Superdex 200 column, eluting at 216–232 ml, i.e., as expected, after the F(ab)₂ fragment but before the small Fc fragments. The D2E7 Fab fragment concentrated to 12.7 mg/ml for crystallization trials, as described below.

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Crystallization of the D2E7 Fab Fragment

The D2E7 Fab fragment (12.7 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method essentially as described above for the F(ab)₂ fragment. Crystals were obtained under

10 many different conditions, as summarized in **Table 2**.

Table 2. Summary of crystallization conditions for the D2E7 Fab fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	4	4	0.1M Tris pH 8.5, 2M (NH ₄) ₂ SO ₄	wispy needles
Hampton 1	10	4	0.2M NH ₄ OAc, 0.1M NaOAc pH 4.6, 30% PEG 4K	wispy needle clusters
Hampton 1	18	4	0.2M Mg(OAc) ₂ , 0.1M Na Cacodylate pH 6.5, 20% PEG 8K	needle clusters
Hampton 1	20	4	0.2M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 4.6, 25% PEG 4K	tiny needle clusters
Hampton 1	32	4	2M (NH ₄) ₂ SO ₄	long, wispy needles
Hampton 1	33	4	4M Na Formate	tiny needle clusters
Hampton 1	38	4	0.1M Hepes pH 7.5	tiny needle clusters
Hampton 1	43	4	30% PEG 1500	tiny needle clusters
Hampton 1	46	4	0.2M Ca(OAc) ₂ , 0.1M Na Cacodylate pH 6.5, 18% PEG 8K	large plate clusters
Hampton 1	47	4	0.1M NaOAc pH 4.6, 2M (NH ₄) ₂ SO ₄	long, wispy needles
Hampton 2	1	4	2M NaCl, 10% PEG 6K	small plate clusters
Hampton 2	2	4	0.01M Hexadecyltrimethylammonium bromide, 0.5M NaCl, 0.01 MgCl ₂	round & irregular plates
Hampton 2	5	4	2M (NH ₄) ₂ SO ₄ , 5% isopropanol	long fiber ropes
Hampton 2	13	4	0.2M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny, wispy needle clusters
Hampton 2	14	4	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M (NH ₄) ₂ SO ₄	tiny needle clusters
Hampton 2	27	4	0.01M ZnSO ₄ , 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Hampton 2	28	4	30% MPD	tiny needle clusters
Hampton 1	4	18	0.1M Tris pH 8.5, 2M (NH ₄) ₂ SO ₄	needle clusters
Hampton 1	9	18	0.2M NH ₄ OAc, 0.1M Na Citrate pH 5.6, 30% PEG 4K	needle clusters
Hampton 1	17	18	0.2M Li ₂ SO ₄ , 0.1M Tris pH 8.5, 30% PEG 4K	long, wispy needles
Hampton 1	32	18	2M (NH ₄) ₂ SO ₄	needle clusters
Hampton 1	33	18	4M Na Formate	tiny needle clusters
Hampton 1	38	18	0.1M Hepes pH 7.5	fiber bundles

Screen	Solution	Temp °C	Condition	Result
Hampton 1	43	18	30% PEG 1500	tiny needle clusters
Hampton 1	47	18	0.1M NaOAc pH 4.6, 2M (NH ₄) ₂ SO ₄	tiny needle clusters
Hampton 2	1	18	2M NaCl, 10% PEG 6K	long, wispy needle clusters
Hampton 2	5	18	2M (NH ₄) ₂ SO ₄ , 5% 2-propanol	tiny needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2M NaCl	long, wispy needles
Hampton 2	13	18	0.2M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny needle clusters
Hampton 2	14	18	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M (NH ₄) ₂ SO ₄	long wispy needles
Hampton 2	27	18	0.01M ZnSO ₄ , 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Wizard I	20	4	0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	28	4	20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl	large orthorhombic plate clusters
Wizard I	31	4	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	wispy needle clusters
Wizard I	39	4	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li ₂ SO ₄	needle clusters
Wizard II	3	4	20% PEG 8K, 0.1M Tris pH 8.5, 0.2M MgCl ₂	large hexagonal or orthorhombic plate cluster in phase sep
Wizard II	4	4	2M (NH ₄) ₂ SO ₄ , 0.1M Cacodylate pH 6.5, 0.2 NaCl	tiny needle clusters
Wizard II	9	4	2M (NH ₄) ₂ SO ₄ , 0.1M phosphate citrate pH 4.2	tiny, wispy needle clusters
Wizard II	28	4	20% PEG 8K, 0.1M MES pH 6, 0.2M Ca(OAc) ₂	tiny needle clusters; large wispy needle clusters
Wizard II	35	4	0.8M NaH ₂ PO ₄ /1.2M K ₂ HPO ₄ , 0.1M NaOAc pH 4.5	tiny fiber bundles
Wizard II	38	4	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li ₂ SO ₄	long wispy needles
Wizard II	47	4	2.5M NaCl, 0.1M Imidazole pH 8, 0.2M Zn(OAc) ₂	tiny needle clusters
Wizard I	6	18	20% PEG 3K, 0.1M Citrate pH 5.5	needle clusters
Wizard I	20	18	0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	27	18	1.2M NaH ₂ PO ₄ /0.8M K ₂ HPO ₄ , 0.1M CAPS pH 10, 0.2M Li ₂ SO ₄	wispy needle clusters
Wizard I	30	18	1.26M (NH ₄) ₂ SO ₄ , 0.1M NaOAc pH 4.5, 0.2M NaCl	wispy needles
Wizard I	31	18	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	tiny needle clusters
Wizard I	33	18	2M (NH ₄) ₂ SO ₄ , 0.1M CAPS pH 10.5, 0.2M Li ₂ SO ₄	fiber bundles
Wizard I	39	18	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li ₂ SO ₄	needle clusters
Wizard II	4	18	2M (NH ₄) ₂ SO ₄ , 0.1M Cacodylate pH 6.5, 0.2 NaCl	needle clusters
Wizard II	9	18	2M (NH ₄) ₂ SO ₄ , 0.1M phosphate citrate pH 4.2	wispy needles
Wizard II	35	18	0.8M NaH ₂ PO ₄ /1.2M K ₂ HPO ₄ , 0.1M NaOAc pH 4.5	tiny needle clusters
Wizard II	38	18	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li ₂ SO ₄	tiny needle clusters

The following conditions (as described in Table 2) produced crystals which can be used for diffraction quality crystals: Hampton 2, 1, 4C, 2M NaCl, 10% PEG 6K,

small plate clusters; Hampton 1 46, 4C, 0.2M Ca Acetate, 0.1M Na Cacodylate, pH 6.5, 18% PEG 8K, large plate clusters; Wizard I, 28, 4C, 20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl, large orthorhombic plate clusters; Wizard II 3, 4C, 20% PEG 8K, 0.1M Tris pH 8.5, 0.2M MgCl₂, lrg hex or orth plate cluster in phase sep.

5

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following

10 claims. The contents of all references, patents and patent applications cited throughout this application are hereby incorporated by reference.